

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/06, A61K 35/28 // C07K 14/705	A1	(11) International Publication Number: WO 99/61587 (43) International Publication Date: 2 December 1999 (02.12.99)
(21) International Application Number: PCT/US99/11975 (22) International Filing Date: 28 May 1999 (28.05.99) (30) Priority Data: 60/087,123 29 May 1998 (29.05.98) US (71) Applicant: OSIRIS THERAPEUTICS, INC. [US/US]; 2001 Aliceanna Street, Baltimore, MD 21231 (US). (72) Inventors: DAVIS-SPROUL, Janice, M.; 1008 Green Acre Road, Towson, MD 21286 (US). MOORMAN, Mark, Aaron; 3575 Shannon Drive, Baltimore, MD 21213-1825 (US). MCNEIL, Renee, Marie; 733 S. Luzerne Avenue, Baltimore, MD 21224 (US). SIMONETTI, Donald, William, Jr.; 3024 Ward Road, P.O. Box 537, Forest Hill, MD 21050 (US). HAMMILL, Lora, Catherine; Apartment F, 7517 Stonecutter Court, Baltimore, MD 21237 (US). CRAIG, Stewart; 200 Dunbeath Court, Timonium, MD 21093 (US). (74) Agents: SEMIONOW, Raina et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: HUMAN CD45+ AND/OR FIBROBLAST + MESENCHYMAL STEM CELLS		
(57) Abstract Human mesenchymal stem cells having the phenotype SH3+, CD45+ can be isolated. These precursor mesenchymal stem cells are useful for treatment of patients in need of mesenchymal stem cells.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Manritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

5

10

HUMAN CD45+ AND/OR FIBROBLAST + MESENCHYMAL STEM CELLS

Background of the Invention

15 This application is based on U.S. provisional application serial no. 60/087,123 filed May 29, 1998.

Mesenchymal stem cells (MSCs) are the formative pluripotential blast cells found *inter alia* in bone marrow, blood, dermis and periosteum that are capable of
20 differentiating into more than one specific type of mesenchymal or connective tissue (*i.e.* the tissues of the body that support the specialized elements; *e.g.* adipose, osseous, stroma, cartilaginous, elastic and fibrous connective tissues) depending upon various influences from bioactive factors, such as cytokines. The potential to differentiate into cells such as osteoblasts and chondrocytes is retained after isolation
25 and expansion in culture; differentiation occurs when the cells are induced *in vitro* under specific conditions or placed *in vivo* at the site of damaged tissue.

Epitopes on the surface of the human mesenchymal stem cells (hMSCs) are reactive with certain monoclonal antibodies known as SH2, SH3 and SH4 described
30 in U.S. Patent No. 5,486,359. These antibodies can be used as reagents to screen and capture the mesenchymal stem cell population from a heterogeneous cell population, such as exists, for example, in bone marrow.

Hematopoietic stem cells (HSCs) are the formative pluripotential blast cells
35 found *inter alia* in bone marrow and peripheral blood that are capable of

differentiating into any of the specific types of hematopoietic or blood cells, such as erythrocytes, lymphocytes, macrophages and megakaryocytes. The expression of a particular antigen or antigens on the cell surface or in the cytoplasm and the intensity of expression indicate the stage of maturation and lineage commitment of the hematopoietic stem cell. Human hematopoietic stem cells (hHSCs) are reactive with certain monoclonal antibodies, such as CD34, recognized as being specific for hematopoietic cells.

Thus, human hematopoietic stem cells and human mesenchymal stem cells have been readily distinguishable by their immunospecific profiles.

Summary of the Invention

The present invention provides a population of human mesenchymal stem cells enhanced in cells that are positive for CD45 antibody markers. As hereinafter indicated, a mesenchymal stem cell is one which is capable of differentiating into more than one specific type of mesenchymal tissue cell. Applicants have provided a population of precursor mesenchymal stem cells ("pre-MSCs") which is positive for CD45. These precursor mesenchymal stem cells can differentiate into the various mesenchymal lineages, for example, the chondrocyte, adipocyte and osteoblast lineages.

In another aspect, the present invention provides a population of human mesenchymal stem cells that are CD45 positive and positive for at least one of the SH2, SH3 or SH4 markers. The mesenchymal stem cells of the present invention are preferably positive for at least the SH3 marker. In another aspect, the precursor mesenchymal stem cells are positive for the SH2 marker.

These precursor mesenchymal stem cells may be obtained using antibodies to markers of mesenchymal and hematopoietic cells. Unexpectedly, it was found that a significant number of cells positive for select markers of mesenchymal stem cells were further characterized as being CD45 positive. CD45 is a marker commonly found on leukocytes and hematopoietic cells and not on cultured mesenchymal stem cells. Although not intending to be bound by any theory, it is believed that the

population of cells of the present invention comprises a precursor cell to more mature, though not committed, mesenchymal stem cells.

The invention further provides a method for recovering an isolated population
5 of CD45+ human mesenchymal stem cells from bone marrow or other mesenchymal
stem cell source of an individual by (i) obtaining bone marrow tissue or other tissue
source of mesenchymal stem cells from a donor; (ii) isolating a population of cells
enriched in mesenchymal stem cells therefrom; and (iii) further selecting CD45+ cells
from the population of human mesenchymal stem cells to obtain a population of
10 mesenchymal stem cells that are enriched in CD45+ mesenchymal stem cells.

In a further aspect, the invention provides a method for recovering an isolated
population of CD45+ human mesenchymal stem cells that are also positive for at
least one of SH2, SH3 or SH4 markers from bone marrow or other mesenchymal
15 stem cell source of an individual by (i) obtaining bone marrow tissue or other source
of mesenchymal stem cell from a donor (ii) isolating a population of cells enriched in
mesenchymal stem cells therefrom; (iii) selecting from the cell population a
population of mesenchymal stem cells that are positive for at least one of SH2, SH3
or SH4 markers; and (iv) further selecting CD45+ cells from the population of human
20 mesenchymal stem cells of step (iii) to obtain a population of mesenchymal stem cells
that are positive for at least one of either SH2, SH3 or SH4 markers and CD45+. In a
preferred embodiment, the CD45 cell population is at least SH3 positive.

Brief Description of the Drawings

25 Figure 1 shows FACSscan histograms for the expression of CD45 cell surface
antigens on the three fractions of human mesenchymal stem cells: Fig. 1A
prefractionation; Fig.1B SH3 selection negative fraction; Fig.1C SH3 selection
positive fraction.

30 Figure 2 shows the results of the calcium deposition assay described in
Example 5.

DETAILED DESCRIPTION OF THE INVENTION

The present invention involves the isolation and enhancement of a subset population of human mesenchymal stem cells. In one embodiment, the present invention provides a population of cells having an SH3+/CD45+ phenotype and
5 which are believed to be a precursor mesenchymal stem cell. The human mesenchymal stem cell population of the present invention is capable of differentiation into the chondrocytic, adipocytic and osteoblast cell lineages.

The mesenchymal stem cells of the present invention can be isolated from
10 peripheral blood or bone marrow. "Isolated" as used herein signifies that the cells are placed into conditions other than their natural environment. The term "isolated" does not preclude the later use of these cells thereafter in combinations or mixtures with other cells. A method of preparing human marrow mesenchymal stem cell cultures has been described in U.S. Pat. No. 5, 486, 359. Several techniques are known to
15 those of skill in the art for the rapid isolation of mesenchymal stem cells. Approaches to mesenchymal stem cell isolation include leucopheresis, density gradient fractionation, immunoselection and differential adhesion separation.

The cells of the present invention are maintained in culture media which can
20 be a chemically defined serum free media or can be a "complete medium", such as Dulbecco's Modified Eagles Medium Supplemented with 10% serum (DMEM). Suitable chemically defined serum free media are described in U.S. Serial No. 08/464,599 and WO96/39487, and "complete media" are described in U.S. Patent No. 5,486,359. Chemically Defined Medium comprises a minimum essential medium
25 such as Iscove's Modified Dulbecco's Medium (IMDM) (Gibco), supplemented with human serum albumin, human Ex Cyte lipoprotein, transferrin, insulin, vitamins, essential and non essential amino acids, sodium pyruvate, glutamine and a mitogen. These media stimulate mesenchymal stem cell growth without differentiation.

30 The mesenchymal stem cells of the present invention isolated from peripheral blood or bone marrow may further be culture-expanded. The cells may be expanded, before or after freezing thereof. The media described herein are also suitable for the culture expansion of the mesenchymal stem cells.

The isolated mesenchymal stem cells of the present invention may further be purified. In a preferred embodiment, "purified" indicates that the cell population contains less than 5% impurities, impurities being for example, cells that are not CD45+. The purified cell population can later be used in combinations or mixtures as
5 is appropriate..

The present invention contemplates any suitable method of employing monoclonal antibodies to separate mesenchymal stem cells from other cells, e.g., recovered from bone marrow. Accordingly, included in the present invention is a
10 method of producing a population of mesenchymal stem cells comprising the steps of providing a cell suspension of tissue containing mesenchymal stem cells; contacting the cell suspension with one or a combination of monoclonal antibodies which recognize an epitope on the mesenchymal stem cells; and separating and recovering
15 from the cell suspension the cells bound by the monoclonal antibodies. The monoclonal antibodies may be linked to a solid-phase and utilized to capture mesenchymal stem cells from tissue samples. The bound cells may then be separated from the solid phase by known methods depending on the nature of the antibody and solid phase.

20 Monoclonal based systems appropriate for preparing the desired cell population include magnetic bead/paramagnetic particle column utilizing antibodies for either positive or negative selection; separation based on biotin or streptavidin affinity; and high speed flow cytometric sorting of immunofluorescent-stained mesenchymal stem cells mixed in a suspension of other cells. Thus, the method of
25 the present invention includes the isolation of a population of hMSCs and enhancement using monoclonal antibodies raised against surface antigens expressed by marrow-derived hMSCs, *i.e.* SH2, SH3 or SH4. Deposits of the cell line cultures identified as SH2, SH3 and SH4 are on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, and are assigned the
30 ATCC accession numbers HB 10743, BH 10744 and HB 10745, respectively. These monoclonal antibodies provide effective probes which can be utilized for identifying, quantifying, and purifying mesenchymal stem cells, regardless of their source in the body.

In one embodiment, the isolation of the cell population of the present invention may comprise utilizing a combination of one or more antibodies that recognize a known marker on mesenchymal stem cells as well as an antibody which recognizes CD45. One method for such preparation of the precursor cells of the present invention is to first select a population of cells expressing a marker identifying mesenchymal stem cells, for example, SH3 or SH2 by immunomagnetic selection of a low density human bone marrow cell sample. Alternatively, it is contemplated that the initial cell selection can be based on the CD45 marker and the cell population be further characterized using the hMSC monoclonal antibodies.

10

In another embodiment, it is contemplated that a cell population can be selected based on the CD14 marker. CD14 is a membrane protein that functions as a receptor for endotoxin (lipopolysaccharide, LPS) and is expressed strongly on the surface of monocytes, but not expressed by myeloid progenitors.

15

Thus, in one aspect, in certain embodiments described herein, the mesenchymal stem cell population Population 1 (Pop 1) is identified by FACS by the relative brightness of immunofluorescent stained antibodies bound thereto as SH2 and SH3 bright/ CD45 dim/ CD14 dim. In comparison, SH2 and SH3 are present on culture expanded MSCs; CD45 is absent on culture expanded MSCs; and CD14 is absent on culture expanded MSCs.

20

In a still further aspect of the invention, a cell population can be selected based on a fibroblast cell surface marker, for example the antifibroblast antibody found on Miltenyi antifibroblast microbeads (Miltenyi catalog # 506-01).

25

It is further contemplated that the hereinabove described methods may be applied to a population of culture-expanded mesenchymal stem cells such that cells having a Pop 1 phenotype may be isolated from the population of culture-expanded mesenchymal stem cells.

30

The present invention is directed to various methods of utilizing the CD45+ human mesenchymal stem cells of the present invention for therapeutic and/or diagnostic purposes. These uses include regenerating mesenchymal tissues which

have been damaged through acute injury, abnormal genetic expression or acquired disease; treating a host having damaged mesenchymal tissue by removal of small aliquots of bone marrow, isolation of their mesenchymal stem cells and treatment of damaged tissue with the CD45+ hMSCs combined with a suitable biocompatible carrier material for delivering the MSCs to the damaged tissue sites; producing various mesenchymal tissues; detecting and evaluating growth factors or inhibitory factors relevant to MSC self-regeneration and differentiation into committed mesenchymal lineages; and developing mesenchymal cell lineages and assaying for factors with mesenchymal tissue development.

10

The hMSCs of the present invention may be used in a variety of ways. For example, the hMSCs can be employed as part of cell replacement therapy. Specifically, the hMSCs can be infused alone or added to bone marrow cells for bone marrow transplant procedures. Other applications also particularly contemplated are orthopedic, are such as augmentation of bone formation. Other applications include, for example, the treatment of osteoarthritis, osteoporosis, traumatic or pathological conditions involving any of the connective tissues, such as a bone defects, connective tissue defects, skeletal defects or cartilage defects. It is also contemplated that exogenous genetic material can be introduced into the cells while *ex vivo*, and that the cells be readministered for production of exogenous proteins *in vivo*. Genetic modification of mesenchymal stem cells is discussed more fully in U.S. Patent No. 5,591,625.

The present invention is not limited to a specific method for recovering the cells. For example, such cells may be isolated by procedures which do not use antibodies, provided that the cells are positive for CD45 and are positive for at least one of SH2, SH3 or SH4, preferably at least SH3, and are capable of differentiating into one or more than one mesenchymal cell lineage, and preferably into most, if not all, of the mesenchymal cell lineages. In a particularly preferred embodiment, the cells are also capable of self renewal. Thus, a human mesenchymal stem cell which is SH3+ and CD45+ in accordance with the invention may be recovered by techniques other than the use of SH3+ and CD45+ antibodies. Thus, the term "human mesenchymal stem cell which is SH3+ and CD45+" means a stem cell that has both

25
30

markers and is capable of differentiating into more than one mesenchymal stem cell lineage.

The following examples are provided to further illustrate and describe the present invention; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Isolation of hMSCs from Human Bone Marrow

Bone marrow aspirates were obtained from three volunteers, donors 271, 281 and 332. Density centrifugation of the bone marrow aspirates was performed using Activated Cell Therapy (ACT) Bouyant Density solution (1.0720 g/ml) in conical tubes (Dendreon, Mountain View CA) and cells were isolated from the light density fraction. Cells were washed and resuspended in Dulbecco's PBS at a concentration of 2×10^7 cells/ml. The cells were incubated with blocking antibody (human IgG 1mg/ml in PBS azide-free) for 10 minutes at 4° C with rotation followed by a 30 minute incubation at 4° C with 1ug/1x10⁷ cells SH3 antibody. Cell from donors 1 and 2 were washed twice with PBS/0.5%BSA and resuspended in PBS to 2×10^7 cells/ml. Dynal beads (washed 3 times with PBS) were added and the suspension was mixed for 30 minutes at 4°C. Bound cells were magnetically separated from unbound cells. Cells from donor 332 were washed 2x with Miltenyi buffer and incubated for 20 minutes at 4°C with mixing with rat anti-mouse IgG2 beads (1ml of microbeads per 5×10^8 cells) (50 nm Miltenyi Biotec, Auburn, CA) and selected according to manufacturer's instructions.

Three cell populations from each donor were analyzed: the start fraction (light density unseparated cells), SH3 selected cells (cells attached to the derivatized magnetic beads) and SH3 unselected cells (cells that did not attach to the derivatized beads). The hematopoietic and mesenchymal stem cell contents of the three samples were assayed as described below.

Cell Numbers. Cell numbers contained in the fractions are shown in Table 1. The SH3 selected fractions contained 4.2, 4.8, and 6.2% of the starting cells. The SH3 unselected fractions yielded 88, 70, and 87% of the starting cells.

Table 1
Cell Numbers (yields)

Fraction	Total cells (x10 ⁶)	% Cell Rec very	% Viability
Donor 271			
Start	262		
SH3 selected	11	4.2	94.2
SH3 unselected	231	88.1	92.8
Donor 281			
Start	217		
SH3 selected	10.5	4.8	90.0
SH3 unselected	150	70.0	99.5
Donor 332			
Start	207		
SH3 Selected	12.8	6.2	
SH3 Unselected	181	87.4	

5 *Colony forming Unit F Assay.* The CFU-F assay measures colonies grown in complete culture media. Nucleated cells were suspended in hMSC medium to a concentration of 2×10^6 cells in 40 mls, and were plated in 100mm tissue culture dishes at 5×10^5 cells per plate. After 14 days cells were fixed with glutaraldehyde and stained with crystal violet. The results are shown in Table 2.

Table 2
CFU-F Assay

10

Fraction	# CFU-F colonies/5x10 ⁴ cells	Total CFU-F (% of total)
Donor 271		
Start	Not done	
SH3 selected	15	3.3×10^3
SH3 unselected	0	0
Donor 281		
Start	1.3	5.77×10^3
SH3 selected	16.7	3.50×10^3 (61%)
SH3 unselected	0.7	2.01×10^3 (35%)
Donor 332		
Start	5.7	2.36×10^4
SH3 Selected	TNTC	
SH3 Unselected	0	0

TNTC = too numerous to count

15 The SH3 selected fraction showed enrichment for colonies when compared to the starting cell sample; in fact, the Miltenyi selected fraction (donor 332) had too many colonies to count. In the SH3 unselected fraction one of 3 CFU-F assays had only 0.7 colonies per 50,000 cells plated, while the remaining 2 cultures had no colony growth.

Table 3
Hematopoietic progenitor cells Total Colonies (% Rec very)

Fraction	BFU-E	CFU-GM	CFU-GEMM
Donor 271			
Start	5.61×10^5	5.30×10^5	0
SH3 selected	0	2.45×10^2 (0.05%)	0
SH3 unselected	3.82×10^5 (68%)	4.72×10^5 (89%)	0
Donor 281			
Start	4.63×10^5	2.62×10^5	0
SH3 selected	1.16×10^2 (0.02%)	1.16×10^2 (0.04%)	0
SH3 unselected	3.08×10^5 (67%)	2.56×10^5 (98%)	0
Donor 332			
Start	6.07×10^5	4.28×10^5	8.28×10^4
SH3 Selected	0	1.28×10^3 (0.3%)	0
SH3 Unselected	5.61×10^5 (92%)	4.40×10^5 (103%)	6.03×10^4 (73%)

- 5 The SH3 unselected fractions contained 89, 98, and 103% of the starting hematopoietic CFU-GM colonies (Table 3). Only 0.04, 0.05, and 0.3% of the starting CFU-GMs were found in the SH3 selected cell fraction (Table 3).

Mesenchymal stem cell culture. 3.2×10^6 cells of Donors 271 and 281 were added into 2 wells of a 6 well plate. The cells were harvested after 13 days in culture.

- 10 The results are shown in Table 4.

For sample donor 332 each well was seeded with 0.8×10^6 cells. On Day 11, based on microscopic examination, two of four SH3 selected cell wells were harvested. All other wells were harvested after 14 days in culture. Results are shown

15 in Table 5.

The results of the MSC cultures showed that the SH3 selected cells expanded with the same or greater efficiency than the starting cell fraction and the harvested cells had the distinctive MSC morphology and phenotype. In MSC complete culture

20 medium after primary culture the cell yield from this SH3 unselected fraction was low (1.3, 3.8, & 1.6%) compared to the starting cell fractions (17.5, 19.4, & 44.5%, respectively).

Table 4
Cell numbers P0 Culture & Harvest

Fraction	Total cells harvested	% Yield
Donor 271		
Start	5.6×10^5	17.5%
SH3 selected	5.7×10^5	17.8%
SH3 unselected	0.42×10^5	1.3%
Donor 281		
Start	6.2×10^5	19.4%
SH3 selected	6.3×10^5	19.7%
SH3 unselected	1.2×10^5	3.8%

Table 5
Donor 332

Fraction	Number of Cells harvested per well	Yield (% of cells seeded)	Appearance
Start	3.56×10^5	44.5	85% confluent
SH3 Selected (Day 11)	3.00×10^5	37.0	85% confluent
SH3 Selected (Day 14)	5.50×10^5	68.8	100% confluent
SH3 Unselected	1.30×10^4	1.6	no spindle shaped cells; cigar shaped or round cells

Cells from the donors 271 and 281 were continued in culture and Passage 1 cells were collected and examined by flow cytometry; the cells were MSCs by morphology and phenotype. Visual observation of these cultures during P0 showed that MSC-like colonies also contained cells with magnetic beads attached. The cells were replated in flasks or wells depending on the total cells available. The cells were harvested after 8 days in culture.

Table 6
Cell Numbers P1 Culture & Harvest

Fraction	# Cells Plated	# Cells Harvested	Fold Increase
Donor 271			
Start	4.3×10^5	2.6×10^6	6.0
SH3 selected	4.3×10^5	1.8×10^6	4.2
SH3 unselected	4.2×10^4	0.13×10^6	3.1
Donor 281			
Start	4.3×10^5	2.3×10^6	5.3
SH3 selected	4.3×10^5	2.0×10^6	4.5
SH3 unselected	1.2×10^5	0.26×10^6	2.3

The cultured cells from the SH3 selected Day 11 harvest were analyzed. The cells were SH2+, SH3+, SH4+ and CD45- , corresponding to a mature cultured mesenchymal stem cell phenotype.

5 *Flow Cytometry Analysis.* The cell fractions of Donor 332 were analyzed using SH3 and CD45 antigen markers. Figure 1 shows the FACS histogram of the CD45 analysis.

Table 7a
Flow Analysis Donor 332

Fraction	Total cells	% SH3 ⁺	Total SH3 ⁺ cells	Yield (%)
Start	207 x 10 ⁶	20.8	43.0 x 10 ⁶	
SH3 Selected	12.8 x 10 ⁶	98.8	12.6 x 10 ⁶	29.4
SH3 Unselected	181 x 10 ⁶	13.6	24.6 x 10 ⁶	57.2

10

Table 7b

Fraction	Total cells	% SH3 ⁺ /CD45 ⁺	Total SH3 ⁺ /CD45 ⁺ Cells	Yield (%)
Start	207 x 10 ⁶	20.4	42.2 x 10 ⁶	
SH3 Selected	12.8 x 10 ⁶	98.4	12.6 x 10 ⁶	29.8
SH3 Unselected	181 x 10 ⁶	12.3	22.3 x 10 ⁶	52.8

The results in Tables 7a and b of the flow analysis of the donor 332 sample demonstrated a SH3 purity of 98.8% with greater than 99.5% of these cells being
15 CD45⁺. The total number of CD45- cells in the sample was 0.42%.

The results indicated that the precursor to the mesenchymal stem cell observed in culture was SH3 positive and CD45 positive and this cell can be isolated using SH3 antibody in conjunction with immunomagnetic beads or other
20 immunoselection methods.

Example 2

MSC Isolation using SH2 cell selection

Biotin-anti-SH2 antibody and rat anti-mouse IgG1 magnetic microbeads
25 were used to isolate two fractions of cells from light-density bone marrow cells: SH2 bound and SH2 unbound. These cell fractions were placed into standard MSC culture conditions to determine the MSC proliferative potential of the cell population contained in these fractions.

Anti-IgG1 microbeads were from Miltenyi Lot # NE7200. VS Column was from Miltenyi Lot # 0231). Pre-separation filters were 30 um from Miltenyi Lot # 55. Miltenyi buffer: phosphate buffered saline pH 7.2 supplemented with 0.5% BSA and 2mM EDTA. Flow staining was performed using the manufacturer's suggested instructions. Flow analysis was performed using either the FACS Calibur or the FACS Vantage. Cell viability and cell number were determined using trypan blue.

Light density cells were isolated from a human bone marrow aspirate (donor #426) using Dendreon (Seattle, WA) solution BDS72 following density centrifugation of bone marrow aspirates. Aliquots were removed as controls for flow analysis and cell culture. The remaining cells were diluted to a cell count of 2×10^7 cells/ml using Miltenyi buffer. Cells were incubated with IgG at 40 ul per ml of cell suspension for 10 minutes at 4°C with mixing. Cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was incubated with the anti-SH2 antibody at 10 ug per 1×10^7 cells for 30 minutes at 4°C with mixing. The cells were washed twice with cold buffer. The cell pellet was resuspended in Miltenyi buffer (80 ul of buffer per 10^7 cells). Anti-IgG1 microbeads were added (20 ul of beads per 10^7 cells). The mixture was incubated at 4°C for 15 minutes with mixing. Miltenyi buffer was added to dilute the mixture and wash the cells. The cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (0.5 ml of buffer per 10^8 cells). The VS column was primed following the manufacturer's instructions. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'SH2 unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'SH2 unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'SH2 bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger used to push the cells into the tube. A fresh column was primed and the cells in the 'SH2 bound' tube were added to the second column. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'SH2 unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this

effluent was added to the 'SH2 unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'SH2 bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger was used to push the cells into the tube. Cell counts and viability assays were performed. Cells from the bound and unbound fractions were stained for flow analysis. Unbound and bound cells were placed in culture. Cells were harvested after 14 days. Cells from the light-density cells and the SH2 bound fraction were stained for flow analysis.

Table 8

SH2 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	2.0×10^8	100
SH2 bound	3.6×10^6	1.8
SH2 unbound	2.3×10^8	113

Table 9

Flow Analysis of the Starting Cell Fractions

Fraction	% SH2+ cells
Light-density cells	23.9
SH2 bound	98.3
SH2 unbound	14.6

Table 10

Microscopic Examination of P0 Cultures on Day 14

Fraction	Appearance
Light-density cells	75% confluent, phase bright cells seen
SH2 Bound	90% confluent, phase bright cells seen
SH2 Unbound	Very small colonies seen

Table 11

P0 Cell Harvests

Fraction	Cells seeded/cm ²	Cells harvested/cm ²	% Yield
Light-density cells	8.0×10^4	1.4×10^4	17
SH2 Bound	8.0×10^4	1.2×10^4	15
SH2 Unbound	8.0×10^4	3.2×10^2	0.4

Table 12

Flow analysis of P0 Cultures

Fraction	% MSC phenotype	% CD45+ cells
Light-density cells	99.5	0.3
SH2 Bound	98.7	0.2

5 The SH2 cell selection of light-density bone marrow cells using the Miltenyi
microbead system yielded less than 2% of the light-density cells in the SH2 bound
fraction. The isolation yielded a cell fraction containing cells that were 98.3%
SH2+. The SH2 bound cells were 90% confluent with spindle-shaped cells after
14 days in culture under MSC standard culture conditions. The culture of the SH2
10 bound cells yielded a population of adherent cells that had an MSC phenotype by
flow analysis and morphology. Very few adherent cells were isolated from the
SH2 unbound cell fraction. These results show that SH2 is an antigen present on
the MSC precursor as well as on the culture-expanded MSC.

Example 3

MSC Isolation of P0 MSC Cultures using CD45 Cell Selection

P0 MSC cultures have been reported to contain a median of 9% CD45+ cells (range, 0.5 to 50%), while the culture-expanded phenotype of MSCs is CD45 negative. A CD45 selection of P0 cells was performed on three donors to
20 determine if MSCs could be cultured from the CD45 bound cell fraction

Anti-CD45 microbeads were from Miltenyi (Lot # NE5848). Large Cell Separation Column was from Miltenyi (Cat # 422-02). Miltenyi buffer: phosphate buffered saline pH 7.2 supplemented with 0.5% BSA and 2mM EDTA. Flow staining was performed using the manufacturer's suggested instructions. Flow analysis was performed using either the FACS Calibur or the FACS Vantage. Cell viability and cell number were determined using trypan blue.

P0 MSCs were derived from light density human bone marrow cells (donors # 394 (0), # 386(0) and # 381(0). Samples of $0.5 - 5.0 \times 10^6$ cells were removed as controls for flow analysis. The remaining cells were diluted to a cell count of 2×10^7 cells/ml using Milenyi buffer; if the count was $< 2 \times 10^7$ cells/ml

this step was skipped. Cells were incubated with IgG at 40 ul per ml of cell suspension for 10 minutes at 4°C with mixing. Cells were centrifuged for 5 minutes at 1100 RPM. The cell pellet was resuspended in Miltenyi buffer (80 ul of buffer per 10^7 cells). Anti-CD45 microbeads were added (20 ul of beads per 10^7 cells). The mixture was incubated at 4°C for 15 minutes with mixing. Miltenyi buffer was added to dilute the mixture and wash the cells. The cells were centrifuged for 10 minutes at 1100 RPM. The cell pellet was resuspended in Miltenyi buffer (0.5 ml of buffer per 10^8 cells). The Large Cell Separation Column was primed following the manufacturer's instructions. The cell/microbead suspension was added to the column and the suspension drained through the column. The effluent was collected as the 'CD45 unbound' fraction. The column was rinsed three times with 0.5 ml of Miltenyi buffer and this effluent was added to the 'CD45 unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'CD45 bound'. One ml of Miltenyi buffer was added to the column and a syringe plunger used to push the cells into the tube. A fresh large cell column was primed following the manufacturer's instructions and the cells in the 'CD45 bound' tube were added to the second column. The cell/microbead suspension was added to the column and the suspension drained through the column. The effluent was collected as the 'CD45 unbound' fraction. The column was rinsed three times with 0.5 ml of Miltenyi buffer and this effluent was added to the 'CD45 unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'CD45 bound'. One ml of Miltenyi buffer was added to the column and a syringe plunger used to push the cells into the tube. Cell counts and viability assays were performed. Cells from the bound and unbound fractions (# 394) and the unbound fraction (# 386 & # 381) were stained for flow analysis. The cells were placed in culture. Cells were harvested and passaged until an adequate number of cells was available for flow analysis.

Table 13

Donor # 381: P0 CD45 Cell selection yields

Fraction	Number of cells	% Yield
P0 cells 381	8.7×10^6	100
CD45 bound	1.4×10^5	1.6
CD45 unbound	4.8×10^6	55

Table 14

Donor # 386: P0 CD45 Cell selection yields

Fraction	Number of cells	% Yield
P0 cells 386	1.2×10^7	100
CD45 bound	2.2×10^5	1.9
CD45 unbound	8.4×10^6	70

Table 15

Donor # 394: P0 CD45 Cell selection yields

Fraction	Number of cells	% Yield
P0 cells 394	1.2×10^8	100
CD45 bound	1.8×10^6	1.5
CD45 unbound	1.1×10^8	92

Table 16

Flow Analysis of the Starting Cell Fractions

Fraction	% CD45+ cells
P0 cells 381	4.7
CD45 unbound 381	1.5
P0 cells 386	1.9
CD45 unbound 386	3.5
P0 cells 394	2.0
CD45 unbound 394	1.4

Table 17

Donor 381(1) Cell Harvests

Fraction	Cells/cm² seeded	Cells/cm² harvested	Days in culture	Fold Expansion
CD45 bound	3.4×10^4	5.8×10^4	12	1.7
CD45 unbound	5.4×10^4	8.3×10^3	7	1.5

Table 18

Donor 381(2) CD45 bound Cell Harvest

Fraction	Cells/cm² seeded	Cells/cm² harvested	Days in culture	Fold Expansion
CD45 bound	2.9×10^3	3.7×10^4	3	12.8

Table 19

Donor 386(1) Cell Harvests

Fraction	Cells/cm² seeded	Cells/cm² harvested	Days in culture	Fold Expansion
CD45 bound	4.4×10^4	7.5×10^4	12	1.7
CD45 unbound	5.4×10^3	1.6×10^4	7	3.0

5

Table 20

Donor 394(1) Cell Harvests

Fraction	Cells/cm² seeded	Cells/cm² harvested	Days in culture	Fold Expansion
CD45 bound	5.3×10^3	1.3×10^4	11	2.5
CD45 unbound	5.3×10^3	3.1×10^4	11	5.0

Table 21

Flow Analysis of Cells Cultured from CD45 Selected P0 MSCs

Fraction	% MSC phenotype	% CD45+
381(2) from CD45 bound	99.2	0.7
386(1) from CD45 bound	99.0	0.8
394(1) from CD45 bound	98.5	1.4

- 10 The CD45 selection of P0 MSCs yielded a cell population which was < 2% of the starting P0 population. Cultured CD45 bound cells isolated from the P0 MSCs yielded MSCs as defined by flow cytometry and morphology. The CD45 unbound cell fraction isolated from the P0 MSCs also yielded MSCs as defined by flow cytometry and morphology. The phenotype of the MSC precursor appeared to be
- 15 CD45 dim.

Example 4**MSC Isolation using CD45 Selection**

- 20 Light-density cells isolated from bone marrow were selected using directly conjugated anti-CD45 Miltenyi microbeads (Miltenyi Lot # NE5848) following the manufacturer's instructions. VS Column was from Miltenyi (Lot # 0231). Pre-separation filters 30 um were from Miltenyi (Lot 55). Miltenyi buffer: phosphate

buffered saline pH 7.2 supplemented with 0.5% BSA and 2mM EDTA. Flow staining was performed using the manufacturer's suggested instructions. Flow analysis was performed using either the FACS Calibur or the FACS Vantage. Cell viability and cell number were determined using trypan blue.

5 Light density cells were isolated from human bone marrow aspirate (donor # 358) using Dendreon solution BDS72 following density centrifugation of the bone marrow aspirates. Aliquots were removed as controls for flow analysis and cell culture. The remaining cells were diluted to a cell count of 2×10^7 cells/ml using Miltenyi buffer. Cells were incubated with IgG at 40 ul per ml of cell
10 suspension for 10 minutes at 4°C with mixing. Cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (80 ul of buffer per 10^7 cells). Anti-CD45 microbeads were added (20 ul of beads per 10^7 cells). The mixture was incubated at 4°C for 20 minutes with mixing. Miltenyi buffer was added to dilute the mixture and wash the cells. The cells were
15 centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (0.5 ml of buffer per 10^8 cells). The VS column was primed following the manufacturer's instructions. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected
20 as the 'CD45 unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'CD45 unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'CD45 bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger was used to push the cells into the tube. A fresh column was primed and the cells
25 in the 'CD45 bound' tube were added to the second column. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'CD45 unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'CD45
30 unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'CD45 bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger was used to push the cells into the tube. Cell counts and viability assays were performed. Cells from the bound and unbound fractions were

stained for flow analysis. Cells were placed in culture. Cells were harvested and counted.

5

Table 22
CD45 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	3.93×10^8	100
CD45 bound	1.84×10^8	47
CD45 unbound	1.37×10^8	35

Table 23

Flow analysis of the Starting Cell Fractions

Fraction	% CD45+ cells
Light-density cells	78.3
CD45 bound	99.0 *
CD45 unbound	51.5

* the staining intensity is significantly enhanced in the CD45 bound cell fraction

10

Table 24

Microscopic Examination of P0 Cultures on Day 14

Fraction	Appearance
Light-density cells	100% confluent, many phase bright cells
CD45 bound	80% confluent, some phase bright cells
CD45 unbound	95% confluent, some phase bright cells

Table 25

P0 Cell Harvests

15

Fraction	Cells/cm² seeded	Cells/cm² harvested	% Yield
Light-density cells	1.6×10^5	5.4×10^4	33
CD45 bound	1.6×10^5	1.9×10^4	12
CD45 unbound	1.6×10^5	4.6×10^4	29

Forty-seven percent of the cells were present in the CD45 bound fraction. This CD45 bound cell population was 99% CD45 positive by flow analysis and was capable of yielding MSCs at P0 as defined by morphology. The CD45

unbound cell fraction was 35% of the total cells selected and was 51.5% CD45 positive. The fluorescence intensity of the CD45 unbound cells was much lower than that of the CD45 bound cells. This cell fraction was also capable of yielding MSCs in P0 culture as defined by morphology. This experiment provides further
5 evidence that the MSC precursor is CD45 positive with a dim staining intensity.

Example 5

MSC Isolation using Fibroblast Cell Selection

Anti-fibroblast microbeads were developed for the separation of cells based
10 on the expression of a fibroblast-specific antigen. Since cultured MSCs have a fibroblastic morphology, the anti-fibroblast microbeads were used to select a fraction of cells from light-density bone marrow cells. Cells that were bound and those that were not bound were placed in standard MSC culture conditions and observed.

15 Anti-fibroblast microbeads were from Miltenyi (Lot # NE630). VS Column was from Miltenyi (Lot # 0231). Pre-separation filters 30 um were from Miltenyi (Lot # 55). Miltenyi buffer: phosphate buffered saline pH 7.2 supplemented with 0.5% BSA and 2mM EDTA. Flow staining was performed following the manufacturer's suggested instructions. Flow analysis was performed using either
20 the FACS Calibur or the FACS Vantage. Cell viability and cell number were determined using trypan blue.

Assays were performed as follows to measure the osteogenic and adipogenic potential of the cells.

Adipogenesis assay. Cells were plated in a 6-well dish (2×10^5 cells/well)
25 in hMSC media. Confluent MSCs were pulse induced with high glucose media containing dexamethasone, insulin, 3-isobutyl-1-methyl-xanthine and indomethacin. At the end of the culture period the plates were fixed with 10% formalin, stained with Oil Red "O" and counterstained with hematoxylin. The formation of lipid vacuoles which stain red were observed and semi-quantitated by
30 percent of well surface area.

Osteogenic calcium deposition assay . Cells were plated in 6-well dishes (3×10^4 cells/well). Wells labeled as "OS" were fed with hMSC media containing supplements of Ascorbic acid-2-phosphate, dexamethasone and β -

glycerophosphate. Wells labeled as "Control" were fed with standard hMSC media. Media changes were performed twice weekly for 14 to 16 days. The increased calcium deposition was measured through semi-quantitative colorimetric assays.

5

Light density cells were isolated from human bone marrow aspirates (donors #373, # 386 & # 421) using Dendreon solution BDS72 following density centrifugation of the bone marrow aspirates. Aliquots were removed as controls for cell culture. The remaining cells were diluted to a cell count of 2×10^7 cells/ml using Miltenyi buffer. Cells were incubated with IgG at 40 ul per ml of cell suspension for 10 minutes at 4°C with mixing. Cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (80 ul of buffer per 10^7 cells). Anti-fibroblast microbeads were added (20 ul of beads per 10^7 cells). The mixture was incubated at room temperature for 30 minutes with mixing. Miltenyi buffer was added to dilute the mixture and wash the cells. The cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (1 ml of buffer per 10^8 cells). The VS column was primed following the manufacturer's instructions. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'fibroblast unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'fibroblast unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'fibroblast bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger was used to push the cells into the tube. A fresh column was primed and the cells in the 'fibroblast bound' tube were added to the second column. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'fibroblast unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'fibroblast unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'fibroblast bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger

was used to push the cells into the tube. Cell counts and viability assays were performed. Cells from donors 373 and 386 were placed in culture. Cells were harvested and passaged. P0 cells from the light density control and the fibroblast bound cultures from donor 373 were stained for flow analysis. P0 cells from donor 386 were placed into the *in vitro* osteogenic differentiation assay and the *in vitro* adipogenic assay. Cultures from the adipogenic assays showed significant adipogenesis of the MSCs.

Table 26

Donor 373 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	6.0×10^7	100
Fibroblast bound	5.6×10^6	9
Fibroblast unbound	4.2×10^7	70

10

Table 27

Donor 386 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	6.0×10^8	100
Fibroblast bound	2.1×10^7	3.5
Fibroblast unbound	4.3×10^8	72

Table 28

Donor 421 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	4.4×10^8	100
Fibroblast bound	2.6×10^7	5.8
Fibroblast unbound	4.2×10^8	94

15

Table 29

Donor 373 Microscopic Examination of P0 Cultures on Day 14

Fraction	Appearance
Light-density cells	80% confluent, phase bright cells seen
Fibroblast bound	50% confluent, phase bright cells seen
Fibroblast unbound	Round cells, debris

Table 30

Donor 386 Microscopic Examination of P0 Cultures on Day 14

Fraction	Appearance
Light-density cells	100% confluent, phase bright cells seen
Fibroblast bound	100% confluent, phase bright cells seen
Fibroblast unbound	Round floating cells, debris

Table 31

Donor 373 P0 Cell Harvests

Fraction	Cells seeded/cm ²	Cells harvested/cm ²	% Yield
Light-density cells	8.1 x 10 ⁴	1.2 x 10 ⁴	15
Fibroblast bound	8.0 x 10 ⁴	5.0 x 10 ³	10
Fibroblast unbound	8.1 x 10 ⁴	3.2 x 10 ¹	0.04

5

Table 32

Donor 386 P0 Cell Harvests

Fraction	Cells seeded/cm ²	Cells harvested/cm ²	% Yield
Light-density cells	1.6 x 10 ⁵	1.6 x 10 ⁴	10
Fibroblast bound	1.6 x 10 ⁵	1.8 x 10 ⁴	11
Fibroblast unbound	1.6 x 10 ⁵	3.9 x 10 ¹	0.02

Table 33

Donor 373 Flow analysis of P0 Cultures

Donor/Fraction	% MSC phenotype	% CD45+ cells
373(0) light-density cells	89.3	10.3
373(0) fibroblast bound cells	85.9	13.8

10

The results of the calcium assay for Donor 386(1), Light-density cell and fibroblast bound cell fractions are shown in Table 39 and Figure 2.

Table 34

Sample ID	Condition	Dilution Factor	Spectrophotometer OD Readings at 575nm			Calcium (ug/well)			Mean	S.D.
			1	2	3	1	2	3		
Light density	Control	20	0.0000	0.0000	0.0000	0.0	0.0	0.0	0.0	0.0
Light density	OS	50	0.1490	0.1625	0.1903	60.4	62.3	66.4	63.0	3.1
Fibro Pos	Control	20	0.0000	0.0000	0.0000	0.0	0.0	0.0	0.0	0.0
Fibro Pos	OS	50	0.1370	0.1554	0.1225	58.6	61.3	56.5	58.8	2.4

The fibroblast bound cell fraction of light density bone marrow cells represented 3–9 % of the starting nucleated cell population and adhered to polystyrene. Under standard MSC culture conditions, the fibroblast bound cell
5 fraction yielded an MSC population as defined by flow cytometry, biological assays and morphology.

Example 6

MSC Isolation using Fibroblast and CD14 Cell Selection

10 Anti-fibroblast microbeads were from Miltenyi (Lot # NE6836). VS Column was from Miltenyi (Lot # 0231). Pre-separation filters 30 um were from Miltenyi (Lot # 55). Miltenyi buffer: phosphate buffered saline pH 7.2 supplemented with 0.5% BSA and 2mM EDTA. Anti-CD14 magnetic beads were from Dynal (Lot # A93900). Flow staining was performed following the
15 manufacturer's suggested instructions. Flow analysis was performed using either the FACS Calibur or the FACS Vantage. Cell viability and cell number were determined using trypan blue.

Light density cells were isolated from a human bone marrow aspirate (donor #391) using Dendreon solution BDS72 following density centrifugation of
20 the bone marrow aspirates. Aliquots were removed as controls for cell culture. The remaining cells were diluted to a cell count of 2×10^7 cells/ml using Miltenyi buffer. Cells were incubated with IgG at 40 ul per ml of cell suspension for 10 minutes at 4°C with mixing. Cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (80 ul of buffer per 10^7 cells).
25 Anti-fibroblast microbeads were added (20 ul of beads per 10^7 cells). The mixture was incubated at 4°C for 15 minutes with mixing. Miltenyi buffer was added to dilute the mixture and wash the cells. The cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (1 ml of buffer per 10^8 cells). The VS column was primed following the manufacturer's instructions.
30 The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'fibroblast unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this

effluent was added to the 'fibroblast unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'fibroblast bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger used to push the cells into the tube. A fresh column was primed and the cells in the 'fibroblast bound' tube were added to the second column. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'fibroblast unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'fibroblast unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'fibroblast bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger used to push the cells into the tube. Cell counts and viability assays were performed. Unbound cells were placed in culture. Bound cells were incubated with Dynal Anti-CD14 magnetic beads at a cell concentration of 2×10^7 cells per ml and a bead concentration of 2×10^7 beads per ml for 1 hour at 4°C with mixing. The cell/bead suspension was placed next to the Dynal hand magnet for 2 minutes. After 2 minutes, the unattached cells were decanted into a tube labeled 'CD14 unbound'. The cell/bead tube was removed from the magnet and 5 ml of buffer added to resuspend the cells. The cell/bead suspension was placed next to the Dynal hand magnet for 2 minutes. After 2 minutes, the unattached cells were decanted into a tube labeled 'CD14 unbound'. The cell/bead tube was removed from the magnet and 5 ml of buffer added to resuspend the cells. The cells that remained attached to beads were counted and placed into culture in two 10 cm^2 wells. The cells in the unbound tube were placed next to the hand magnet for 2 minutes. Unattached cells were decanted, counted and plated into one 10 cm^2 well. Cells were harvested after 14 days. Cells cultured from the light-density cells and the Fibroblast bound/CD14 bound fractions were stained for flow analysis.

Table 35

Fibroblast Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	3.35×10^8	100
Fibroblast bound	1.2×10^7	3.4
Fibroblast unbound	3.51×10^8	105

Table 36

CD14 Cell selection yields

Fraction	Number of cells	% Yield
Fibroblast bound	9×10^6	100
CD14 bound	3.4×10^6	38
CD14 unbound	1.9×10^6	21

Table 37

Microscopic Examination of P0 Cultures on Day 14

Fraction	Appearance
Light-density cells	100% confluent, phase bright cells seen
Fibroblast unbound	Round cells as singles and clumps
Fibroblast bound/CD14 bound	100% confluent with beads attached to some spindle shaped cells
Fibroblast bound/CD14 unbound	Round cells in singles and clumps

Table 38

P0 Cell Harvester

Fraction	Cells seeded/cm²	Cells harvested/cm²	% Yield
Light-density cells	1.6×10^5	7.6×10^4	47
Fibroblast unbound	1.6×10^5	1.6×10^2	0.1
Fibroblast bound/CD14 bound	1.7×10^5	4.0×10^4	23
Fibroblast bound/CD14 unbound	1.9×10^5	1.0×10^3	0.5

Table 39

Flow analysis of P0 Cultures

Fraction	% MSC phenotype	% CD45+ cells
Light-density cells	98.1	1.0
Fibroblast bound/CD14 bound	98.3	0.3

In order to further define the phenotype of the MSC precursor, a sequential cell selection was performed using Miltenyi microbeads to select fibroblast bound cells, followed by the use of Dynal magnetic beads to isolate CD14 bound cells from the fibroblast bound fraction. This is possible since the size of the microbead is too small to interfere with the Dynal selection.

Using this technique, the fibroblast bound/CD14 bound cell fraction was approximately 1.3% of the starting light-density cell population. The fibroblast bound/ CD14 bound cells, when placed in standard MSC culture conditions, adhered to polystyrene flasks and after 14 days of culture, yielded a MSC population as defined by flow analysis and morphology.

Based on this single selection, it would appear that the MSC precursor is a fibroblast+, CD14+ cell. This is unexpected since the culture expanded MSC is a fibroblast+, CD14 negative cell.

Example 7

MSC Isolation using Fibroblast Cell Selection and Flow Sorting

Anti-fibroblast microbeads were from Miltenyi (Lot # NE7105). VS Column was from Miltenyi (Lot # 0231). Pre-separation filters 30 um were from Miltenyi (Lot # 55). Miltenyi buffer: phosphate buffered saline pH 7.2 supplemented with 0.5% BSA and 2mM EDTA. Flow staining was performed following the manufacturer's suggested instructions. Flow analysis was performed using either the FACS Calibur or the FACS Vantage. Flow sorting was performed on the FACS Vantage. Determination of cell viability and cell number was done using trypan blue). Osteogenic potential was measured using the method described in Example 5 and with an *in vivo* osteogenic cube assay as described for example in U.S. Pat. No. 5,486,359. Adipogenic assays were performed according to methods described in Example 5.

The chondrogenesis assays was performed as follows. Cells were pelleted in a 15ml conical tube (2.5×10^5 cells/pellet) in chondrogenic media, consisting of high glucose, dexamethasone and TGF- β 3. The pellets were submitted to histology for embedding, thin sectioning and histochemical staining. The presence

of chondrocytes was detected using Toluidine Blue, which stains for proteoglycans and with an antibody specific for type II collagen.

Light density cells were isolated from human bone marrow aspirate (donors # 401 and # 438) using Dendreon solution BDS72 following density centrifugation of the bone marrow aspirates. Aliquots were removed as controls for flow analysis and cell culture. The remaining cells were diluted to a cell count of 2×10^7 cells/ml using Miltenyi buffer. Cells were incubated with IgG at 40 ul per ml of cell suspension for 10 minutes at 4°C with mixing. Cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (80 ul of buffer per 10^7 cells). Anti-fibroblast microbeads were added (20 ul of beads per 10^7 cells). The mixture was incubated at room temperature for 30 minutes with mixing. Miltenyi buffer was added to dilute the mixture and wash the cells. The cells were centrifuged for 10 minutes at 1000 RPM. The cell pellet was resuspended in Miltenyi buffer (1 ml of buffer per 10^8 cells). The VS column was primed following the manufacturer's instructions. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'fibroblast unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'fibroblast unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'fibroblast bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger was used to push the cells into the tube. A fresh column was primed and the cells in the 'fibroblast bound' tube was added to the second column. The pre-filter was primed following the manufacturer's instructions. The cell/microbead suspension was added to the pre-filter and the suspension drained through the column. The effluent was collected as the 'fibroblast unbound' fraction. The column was rinsed twice with 3 ml of Miltenyi buffer and this effluent was added to the 'fibroblast unbound' fraction. The column was removed from the magnet and placed over a tube labeled 'fibroblast bound'. Five ml of Miltenyi buffer was added to the column and a syringe plunger was used to push the cells into the tube. Cell counts and viability assays were performed.

Cells from the fibroblast unbound fraction were stained for flow analysis. Cells from the fibroblast bound fraction were incubated with anti-SH3, anti-CD45

and anti-CD14. Cells were sorted: Donor 401 was sorted into two groups: population 1 and population 2. Donor 438 was sorted into three groups: population 1, population 1a and population 2. Cells from the fibroblast unbound fraction (Donor 401 only) and the sorted populations were placed in culture. P0 cells were harvested and passaged. P1 cells from the light-density and population 1 cultures on donor # 401 were stained for flow cytometry and placed in the *in vivo* cube assay. P1 cells for donor # 438 were passaged. P2 cells from the light density, population 1 and population 1a cultures on donor # 438 were stained for flow cytometry and placed in the *in vitro* biological assays (osteogenic, adipogenic and chondrogenic).

Table 40

Donor 401 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	6.6×10^8	100
Fibroblast bound	2.9×10^7	4.4
Fibroblast unbound	5.4×10^8	82

Table 41

Donor 438 Cell selection yields

Fraction	Number of cells	% Yield
Light-density cells	8.5×10^8	100
Fibroblast bound	2.0×10^7	2.4
Fibroblast unbound	7.9×10^8	93

Table 42

Fibroblast Bound Cells after Flow Sorting

Fraction	Surface Phenotype Flow Profile	Number of Cells
Donor 401 Population 1	SH3+ bright; CD14 dim	1.1×10^4
Donor 401 Population 2	SH3+ bright; CD14 bright	2.0×10^4
Donor 438 Population 1	SH3+ bright; CD14 dim	1.5×10^4
Donor 438 Population 1a	SH3+ bright; CD45 dim	1.5×10^4
Donor 438 Population 2	SH3+ bright; CD14 bright	4.8×10^5

Table 43

Donor 401: Microscopic Examination of P0 Cultures on Day 12

Fraction	Appearance
Light-density cells	60% confluent, phase bright cells seen
Fibroblast unbound	Round cells with rare spindle shaped cells seen
Fibroblast bound Population 1	100% confluent, phase bright cells seen
Fibroblast bound Population 2	Round cells with rare spindle shaped cells seen

5

Table 44

Donor 438: Microscopic Examination of P0 Cultures on Day 14

Fraction	Appearance
Light-density cells	90 % confluent, phase bright cells seen
Fibroblast bound Population 1	100 % confluent, phase bright cells seen
Fibroblast bound Population 1a	100% confluent, phase bright cells seen
Fibroblast bound Population 2	Debris

Table 45

Donor 401: P0 (Day 12 cultures) Cell Harvests

Fraction	Cells Seeded/cm²	Cells Harvested/cm²	% Yield
Light-density cells	8.0×10^4	1.7×10^4	21
Fibroblast unbound	8.0×10^4	6.7×10^2	0.08
Population 1	8.9×10^2	1.7×10^3	196
Population 2	1.7×10^3	7.1×10^2	42

10

Table 46

Donor 438: P0 (Day 14 cultures) Cell Harvests

Fraction	Cells Seeded/cm²	Cells Harvested/cm²	Fold Expansion
Light-density cells	8.0×10^4	4.5×10^4	0.6
Population 1	6.0×10^2	4.2×10^4	70
Population 1a	6.0×10^2	4.6×10^4	77
Population 2	9.6×10^3	0	0

Table 47

P1 (Day 7 cultures) Cell Harvests

Fraction	Cells Seeded/cm²	Cells Harvested/cm²	Fold Expansion
401 Light-density cells	6.7×10^3	3.6×10^4	5
401 Population 1	0.3×10^3	2.5×10^4	90.0
438 Light-density cells	6.1×10^3	2.3×10^4	4
438 Population 1	5.7×10^3	2.1×10^4	4
438 Population 1a	6.2×10^3	2.6×10^4	5

Table 48

Flow analysis of P1 Cultures Donor 401(1)

Fraction	% MSC phenotype	% CD45+
Light-density cells	99.2	0.2
Population 1	99.8	<0.1

5

Table 49

In vivo Cube Assay Results Donor 401(1)

Fraction	Average Score for 3 cubes
Light-density cells	0.3
Population 1	0.5
Negative Control	0

Table 50

Donor 438: P2 (Day 7 cultures) Cell Harvest

Fraction	Cells seeded/cm²	Cells Harvests/cm²	Fold Expansion
Light-density cells	5.4×10^3	2.1×10^4	3.8
Population 1	5.3×10^3	2.4×10^4	4.5
Population 1a	5.3×10^3	2.2×10^4	4.2

10

Table 51

Flow analysis of P2 Cultures Donor 438(2)

Fraction	% MSC phenotype	% CD45+
Light-density cells	99.9	< 0.1
Population 1	99.9	< 0.1
Population 1a	99.9	< 0.1

Table 52

Donor 438(2): Adipogenesis Results

Fraction	% Area with Vacuoles		
	Adipogenic Medium	Adipogenic Maintenance Medium	Human MSC Medium
Light-density cells	40	0	0
Population 1	40	0	0
Population 1a	40	0	0

All samples measured positive as evaluated by established acceptance criteria.

5

Table 53

Donor 438(2): Osteogenesis Results

Fraction	Calcium ug/dish		Calcium ug/million cells	
	Control	OS	Control	OS
Light-density cells	1.0	16.2	5.1	12.9
Population 1	1.1	18.0	7.2	15.1
Population 1a	1.1	14.4	5.8	12.8

All samples measured positive as evaluated by established acceptance criteria.

Table 54a

10

Donor 438(2): Chondrogenesis Results; Toluidine Blue Results

Fraction	Toluidine Blue (%)			
	Day 7	Day 14	Day 21	Day 28
Light-density cells	60	40	70	100
Population 1	70	100	90	100
Population 1a	90	100	100	100

Table 54b

Donor 438(2): Chondrogenesis Results; Collagen Type II Results

Fraction	Anti-Collagen Type II (%)			
	Day 7	Day 14	Day 21	Day 28
Light-density cells	0	0	30	50
Population 1	0	100	70	80
Population 1a	0	90	100	80

Table 54c

Donor 438(2): Chondrogenesis Results; Pellet Size

Fraction	Diameter (millimeters)			
	Day 7	Day 14	Day 21	Day 28
Light-density cells	1.0	0.8	0.9	1.0
Population 1	0.8	1.2	1.5	1.5
Population 1a	1.0	1.4	1.6	1.5

All samples measured positive as evaluated by established acceptance criteria.

5

To further define the phenotype of the MSC precursor, fibroblast bound light-density bone marrow cells were stained with anti-SH3 and anti-CD14 and sorted using flow cytometry.

This resulted in the sorting of population 1, population 1a and population 2.

- 10 All three of these populations were SH3+ bright. Population 1 was CD14+ with a dim staining intensity. Population 1a was CD45+ with a dim staining intensity. Population 2 was CD14+ with a bright staining intensity. MSCs were cultured from Population 1 and Population 1a cell fractions, as confirmed from flow analysis and *in vitro* and *in vivo* biological assays. It was estimated that one
- 15 Population 1/1a cell is present in 10^5 light-density bone marrow cells.

CLAIMS

1. An isolated population of human mesenchymal stem cells comprising human mesenchymal stem cells which are CD45+.
- 5 2. The isolated population of human mesenchymal stem cells of claim 1 wherein the human mesenchymal stem cells which are CD45 + are further reactive with at least one monoclonal antibody selected from the group consisting of SH2 antibody, SH3 antibody and SH4 antibody.
- 10 3. The isolated cell population of claim 2 wherein the human mesenchymal stem cells are SH3+and CD45+.
4. The isolated cell population of claim 2 wherein the human mesenchymal stem cells are SH2+ and CD45+.
- 15 5. A composition comprising an isolated population of human mesenchymal stem cells which bear a fibroblast surface marker.
6. The composition of claim 5 wherein the human mesenchymal stem cells are
20 also CD45+.
7. The composition of claim 6 further comprising CD14+ cells.
8. A process of isolating a population of human mesenchymal stem cells which
25 are CD45+, comprising
 - (a) obtaining an enriched population of human mesenchymal stem cells; and
 - (b) selecting from the cell population of (a) cells that are CD45+.
9. A process of isolating a population of human mesenchymal stem cells which
30 are SH3+ and CD45+, comprising
 - (a) obtaining an enriched population of human mesenchymal stem cells that are SH3+; and
 - (b) selecting from the cell population of (a) cells that are CD45+.

10. A process of isolating a population of human mesenchymal stem cells which are SH2+ and CD45+, comprising
- (a) obtaining an enriched population of human mesenchymal stem cells that are SH2+; and
 - 5 (b) selecting from the cell population of (a) cells that are CD45+.
11. A process of isolating a population of human mesenchymal stem cells which are fibroblast+, comprising
- (a) obtaining an enriched population of human mesenchymal stem
10 cells; and
 - (b) selecting from the cell population of (a) cells that are fibroblast+.
12. The method of claim 11, further comprising selecting from the fibroblast+
15 cell population cells that are SH3+.
13. The method of claim 12, further comprising selecting from the fibroblast+/SH3+ cell population cells that are CD45+.
- 20 14. A method for treating a patient, comprising administering human mesenchymal stem cells which are CD45+ to a patient in need thereof.
15. The method of claim 14 wherein the human mesenchymal stem cells are SH3+ and CD45+.
- 25 16. The method of claim 14 wherein the human mesenchymal stem cells are SH2+ and CD45+.
17. The method of claim 14 wherein the mesenchymal stem cells are
30 administered to generate bone formation.
18. The method of claim 14 wherein the mesenchymal stem cells are administered to treat or repair a connective tissue defect in the patient.

19. The method of claim 18 wherein the defect is a bone defect.
20. The method of claim 18 wherein the defect is a cartilage defect.
- 5 21. The method of claim 14 wherein the mesenchymal stem cells are administered to enhance hematopoietic stem or progenitor cell engraftment in an individual in need thereof.
22. A method for treating a patient, comprising administering human
10 mesenchymal stem cells which are fibroblast+ to a patient in need thereof.
23. Isolated CD45+ human mesenchymal stem cells transfected with exogenous genetic material encoding a protein to be expressed.
- 15 24. Isolated fibroblast+ human mesenchymal stem cells transfected with exogenous genetic material encoding a protein to be expressed.

FIG.1A
Pre-fractionation

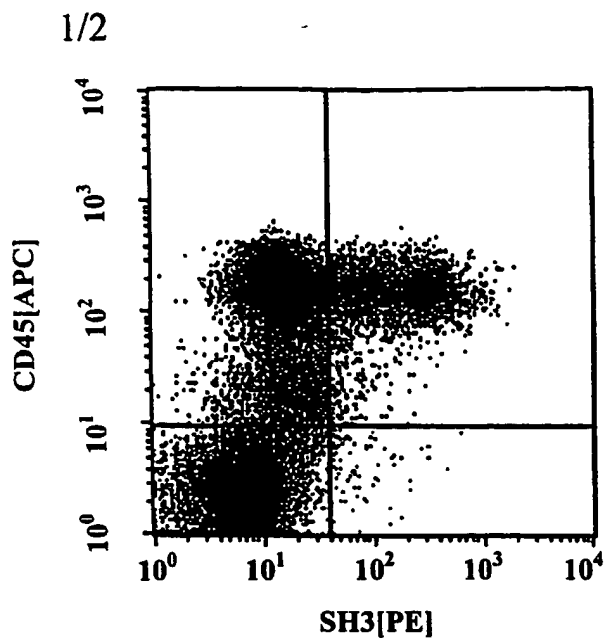


FIG.1B
SH3 negative fraction

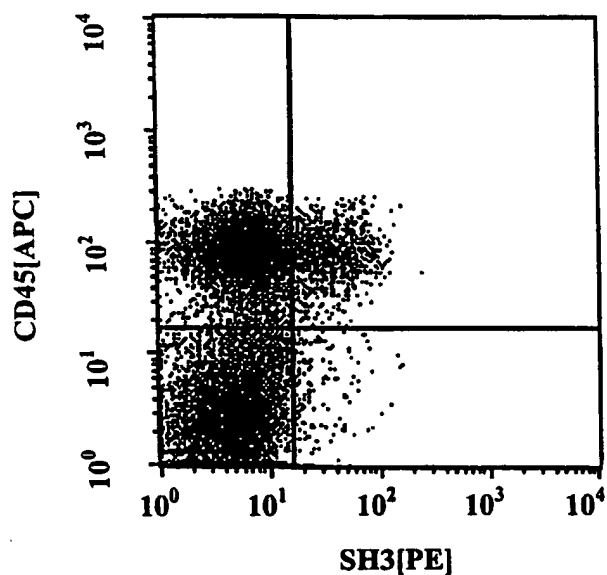
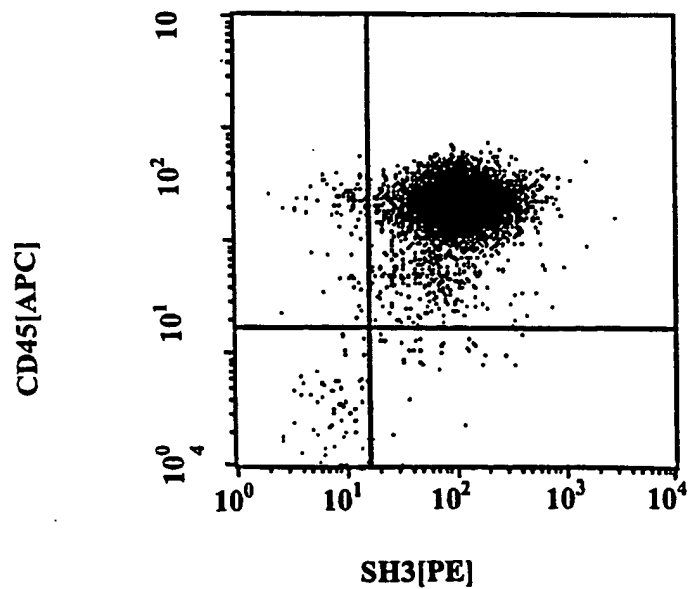
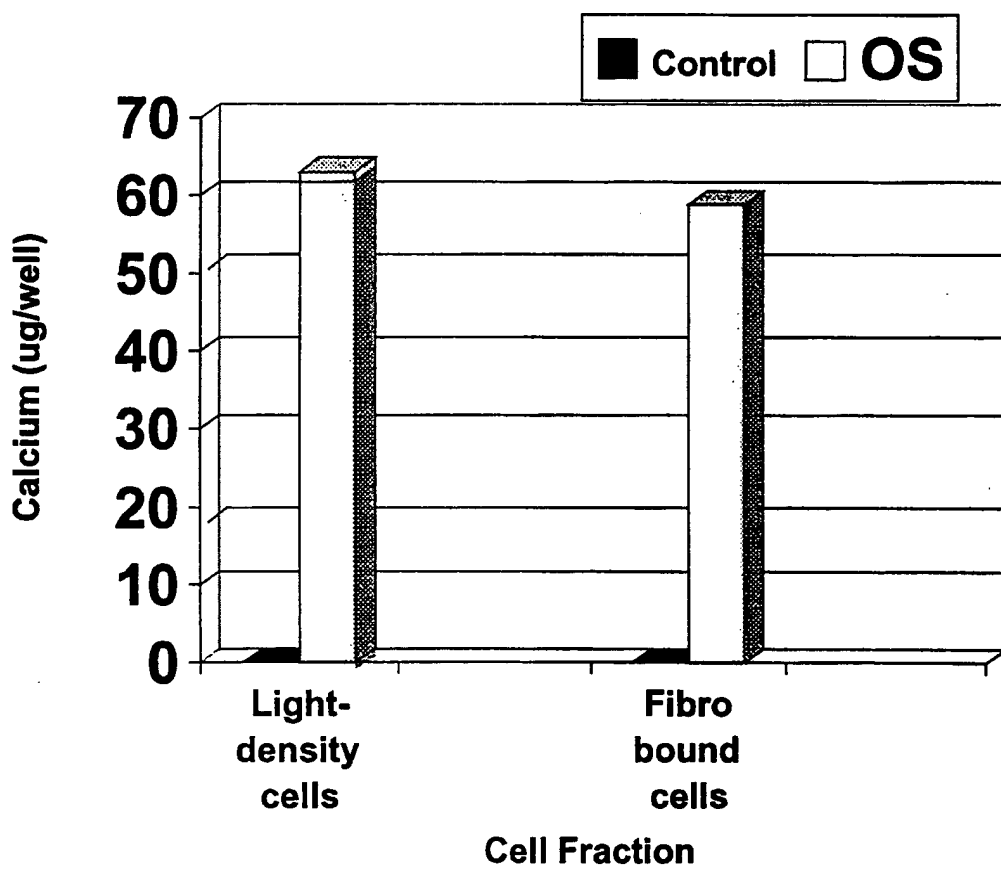


FIG.1C
SH3 positive fraction



2/2

FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/11975

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/06 A61K35/28 //C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W0 96 38482 A (OSIRIS THERAPEUTICS INC) 5 December 1996 (1996-12-05) page 13, paragraph 3 - page 17, paragraph 1	1-7
X	US 5 654 186 A (CERAMI ANTHONY ET AL) 5 August 1997 (1997-08-05) column 2, line 45 - column 3, line 20	1-24

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 August 1999

Date of mailing of the international search report

23/08/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Chakravarty, A

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 99/11975

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9638482 A	05-12-1996	US 5837539 A AU 5958996 A	17-11-1998 18-12-1996
US 5654186 A	05-08-1997	US 5804446 A US 5846796 A	08-09-1998 08-12-1998